British Journal of Pharmacology (1997) 120, 239–246

Preliminary characterization of the role of protein serine/threonine phosphatases in the regulation of human lung mast cell function

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1 Okadaic acid, a cell permeant inhibitor of protein serine/threonine phosphatases (PPs), attenuated the IgE-dependent release of mediators from human lung mast cells (HLMC). The concentration of okadaic acid required to inhibit by 50% (IC_{50}) the IgE-dependent release of histamine was 0.2 μM. Okadaic acid also inhibited the IgE-mediated generation of prostaglandin D_2 (PGD_2) and sulphopeptidoleukotrienes (sLT) with IC_{50} values of 0.2 μM and 0.6 μM respectively.

2 The IgE-mediated generation of histamine, PGD_2 and sLT was inhibited by okadaic acid and two analogues of okadaic acid, okadaol and okadaone, with the following rank order of activity; okadaic acid > okadaol > okadaone. This order of activity for the inhibition of mediator release parallels the activity of these compounds as inhibitors of isolated PPs.

3 Extracts of HLMC liberated ^{32}P from radiolabelled glycogen phosphorylase and this PP activity was inhibited by the PP inhibitors (all at 3 μM), okadaic acid ($73 \pm 4\%$ inhibition, $P < 0.0005$), okadaol ($26 \pm 7\%$ inhibition, $P < 0.05$) and okadaone ($8 \pm 7\%$ inhibition, $P = 0.52$). The rank order of activity of okadaic acid > okadaol > okadaone parallels the activity of these compounds as inhibitors of isolated PPs.

4 Dephosphorylation of radiolabelled glycogen phosphorylase by extracts of HLMC was inhibited by $15 \pm 3\%$ ($P < 0.001$) by a low (2 nM) concentration of okadaic acid and by $88 \pm 4\%$ ($P < 0.0005$) by a higher (5 μM) concentration of okadaic acid. Because 2 nM okadaic acid may act selectively to inhibit PP2A whereas 5 μM okadaic acid inhibits both PP1 and PP2A, these data suggest that both PP1 and PP2A are present in HLMC.

5 Inhibitor 2, a PP1-selective inhibitor, attenuated ($71 \pm 3\%$ inhibition, $P < 0.05$) PP activity in extracts of HLMC suggesting that HLMC contain PP1 and that it may constitute 71% of the phosphorylase PP activity in extracts of HLMC.

6 Radiolabelled casein, a PP2A-restricted substrate, was dephosphorylated by extracts of purified HLMC and this activity was inhibited ($81 \pm 8\%$ inhibition, $P < 0.005$) by 2 nM okadaic acid suggesting that PP2A is resident in HLMC.

7 Collectively, these data suggest that both PP1 and PP2A are resident in HLMC. However, although the data suggest that okadaic acid regulates responses in HLMC by interacting with PPs, it has not been possible to determine whether either PP1 or PP2A or both PPs are involved in the okadaic acid-induced inhibition of mediator release from HLMC.

Keywords: Mast cells; mediator release; phosphatases; dephosphorylation; okadaic acid

Introduction

A large body of evidence indicates that phosphorylations and dephosphorylations are important in the regulation of cellular activity (Benhamou & Siraganian, 1992; Hamawy *et al*., 1995). Antigenic activation of rodent mast cells has been shown to lead to both tyrosine and serine/threonine phosphorylations of a variety of known and unknown proteins (Paolini *et al*., 1991). How far these studies on rodent mast cells mirror the situation in human mast cells has not been established. Although no direct demonstration has been provided to show that phosphorylation states change in activated human mast cells, indirect evidence, based on studies with protein kinase inhibitors which attenuate the responses of human mast cells, suggests that phosphorylations are important (Massey *et al*., 1991; Lavens *et al*., 1992). Less information is available on the role that dephosphorylations may play in the regulation of mast cell responses. However, the recent identification of a number of phosphatase (PP) inhibitors, some of which are cell permeant, should help to delineate the importance of dephosphorylations in the mediation of mast cell responses.

Four major classes of serine/threonine PP have been identified which have been broadly classified as type 1 PPs (PP1) which dephosphorylate the β subunit of phosphorylase kinase and type 2 PPs (PP2A, PP2B and PP2C) which dephosphorylate the α subunit of phosphorylase kinase (Cohen, 1989; Cohen & Cohen, 1989; Shenolikar, 1994). Additionally, the type 1 PPs are sensitive to the thermostable proteins, inhibitor 1 and inhibitor 2, whereas the type 2 PPs are unaffected. Both inhibitor 1 and inhibitor 2 are endogenous cytosolic inhibitors of PP1 and inhibit PP1 at nanomolar concentrations (Cohen & Cohen, 1989). The more recent identification of okadaic acid as an inhibitor of PPs has proved particularly useful as a complementary tool in the classification of PPs (Cohen *et al*., 1989; 1990). Okadaic acid inhibits isolated PP2A at low (IC_{50}, 0.1 nM) concentrations whereas higher (IC_{50}, 10 nM) concentrations are required to inhibit PP1 (Cohen *et al*., 1989). Okadaic acid is considerably less potent as an inhibitor of PP2B (IC_{50}, 5 μM) and is inactive against PP2C (Cohen *et al*., 1989; 1990). More recent information indicates that okadaic acid, at nanomolar concentrations, also inhibits a number of novel PPs, PP3, PP4 and PP5 (Honkanen *et al*.,

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1991; Brewis *et al*., 1993; Chen *et al*., 1994). This finding would appear to complicate ascribing an okadaic acid-sensitive activity to a given PP. However, PP3, PP4 and PP5 seem to be involved in the regulation of the cell cycle and a role for these PPs in fully-differentiated cells would seem less likely.

A particularly attractive feature of okadaic acid is that it is cell permeant (Haystead *et al*., 1989; Hardie *et al*., 1991). Okadaic acid has been used in a number of different cell types to implicate PPs and thereby dephosphorylations in the regulation of cellular processes (Cohen *et al*., 1990). Our own previous studies have shown that okadaic acid inhibits the stimulated release of mediators from human lung mast cells (HLMC) suggesting that PPs may regulate HLMC responses (Peachell & Munday, 1993). Similar studies in rat peritoneal mast cells (Takei *et al*., 1993; Estevez *et al*., 1994), rat basophilic leukaemia cells (Sakamoto *et al*., 1994) and human basophils (Botana & MacGlashan, 1993; Peirce *et al*., 1996) have also been reported. In the present work we have attempted to characterize PP activities in HLMC and to determine whether okadaic acid inhibits HLMC responses by interacting with PPs.

Methods

Isolation of HLMC

Mast cells were isolated from human lung tissue by a modification of previously described methods (Ali & Pearce, 1985). Macroscopically normal tissue from lung resections of patients with carcinoma was stripped of its pleura and chopped vigorously for 15 min with scissors in a small volume of −PBS. The chopped tissue was washed over a nylon mesh (100 μm pore size; Cadisch and Sons, London) with 0.5–1 l of −PBS to remove lung macrophages. The tissue was reconstituted in PBS (10 ml g^{-1} tissue) containing collagenase Ia (350 u ml^{-1} of PBS) and agitated (90 min) by a water-driven magnetic stirrer immersed in a water bath at 37°C. The supernatant (containing some HLMC) was separated from the tissue by filtration over nylon mesh. The collagenase-treated tissue was then reconstituted in a small volume of PBS buffer and disrupted mechanically with a syringe. The disrupted tissue was then washed over nylon gauze with PBS (300–600 ml). The pooled filtrates were centrifuged ($120 \times g$, 8 min) at room temperature (RT), the supernatant discarded and the pellets reconstituted in PBS (100 ml). The pellet was washed a further two times. HLMC were visualized by microscopy by use of an alcian blue stain (Gilbert & Ornstein, 1975). Of the total cells, 3–13% were HLMC. This method yielded approximately 2 to 9×10^5 HLMC g^{-1} tissue. On occasion, HLMC were purified by countercurrent elutriation (CCE) by a Beckman J6B centrifuge fitted with a model JE-5.0 elutriator head, followed by flotation over discontinuous Percoll density gradients, methods which have been described in detail elesewhere (Schulman *et al*., 1982; Ishizaka *et al*., 1983). HLMC purified by CCE were used in experiments in which the effect of PP inhibitors on cell function (e.g. mediator release) was assessed. CCE generated HLMC at purities of between 70 and 93%.

Alternatively, HLMC were purified by immunomagnetic bead separations. The method involved positive selection of HLMC by using c-kit receptor as the surface marker for isolation. Impure preparations of HLMC were subjected to a simple Percoll density (40:60%) centrifugation. Cells at the 40:60% interface were harvested, washed in PBS-EDTA resuspended in PBS-EDTA (2×10^6 HLMC per 100 μl) and incubated (1 h) with monoclonal (IgG_1) mouse anti-human c-kit receptor (50 μg ml^{-1}). Cells were then washed twice with PBS-EDTA over ice and incubated (30 min) in PBS-EDTA (2×10^6 HLMC per 100 μl) containing Dynal magnetic beads coated with a rat anti-mouse IgG_1 antibody at a ratio of beads to HLMC of 4 to 1. The magnetic fraction was harvested, with a Dynal MPC-1 magnet, and washed (5×1 ml) with ice-cold PBS-EDTA and the magnetically adherent cells counted with alcian blue to determine HLMC purities (Gilbert & Ornstein, 1975). This fraction typically contained $1-3 \times 10^6$ HLMC at between 89 and 99% purity. These highly purified cells were employed in some of the PP assays. Whilst it is possible that this method of purification may activate the cells in some way, it should be stressed that cell isolation was performed on ice and in the presence of EDTA (1 mM) and cells were immediately subjected to hypotonic lysis and extracts stored at −80°C for use at a later date.

Mediator release

Mediator release experiments were performed in +PBS buffer. HLMC were incubated with or without PP inhibitors for time periods as indicated in the text before activation. In some experiments, especially in situations where long (21 h) incubation times were involved, cells were incubated with a given PP inhibitor in RPMI 1640 buffer supplemented with gentamicin (50 μg ml^{-1}) and penicillin-streptomycin (10 μg ml^{-1}), typically in a volume of 0.5 ml, and maintained in a CO_2 incubator at 37°C. After this incubation, the cells were pelleted ($120 \times g$, RT, 4 min) and washed in +PBS buffer before challenge with stimulus. Mediator release from HLMC (2 to 3×10^4 HLMC per sample) was initiated immunologically (anti-IgE) or with the calcium ionophore A23187 and secretion was allowed to proceed for 30 min at 37°C after which time the cells were pelleted by centrifugation ($160 \times g$, RT, 4 min). Histamine released into the supernatant was determined by the modified (Ennis, 1991) automated fluorometric method of Siraganian (1974) and, when appropriate, an aliquot of the supernatant was removed and stored frozen for sLT and PGD_2 analysis by enzyme immunoassay (EIA). Total histamine content was determined by lysing aliquots of the cells with perchloric acid at a final concentration of acid of 1.6%. Cells incubated in buffer alone served as a measure of spontaneous histamine release which ranged from 2–8% of the total histamine content. Long-term (21 h) incubation of cells in the presence of okadaic acid (1 μM) did not affect either the total number of HLMC recovered, the total histamine content or the spontaneous histamine release compared to untreated cells. Histamine release was thus expressed as a percentage of the total histamine content after subtracting the spontaneous histamine release. All experiments were performed in duplicate.

Phosphatase assays

Lysates of purified HLMC preparations, for use in PP assays, were prepared as described elsewhere (Fruman *et al*., 1992). Purified cells were resuspended in hypotonic lysis buffer (5×10^6 HLMC in 100 μl) and disrupted by three cycles of freeze-thawing. Following centrifugation ($13000 \times g$, 10 min), supernatants were snap frozen in liquid nitrogen and stored at −80°C for use at a later date. Radiolabelled phosphorylase a was prepared (Resink *et al*., 1983) from phosphorylase b (5 mg ml^{-1}) by incubation in the presence of phosphorylase kinase (200 u ml^{-1}), $MgCl_2$ (4 mM), $CaCl_2$ (0.1 mM), [γ^{32}P]-ATP (0.2 mM; specific activity 0.75 to 1.3×10^6 c.p.m. $nmol^{-1}$) at 37°C for 2 h. Unreacted [γ^{32}P]-ATP was removed by extensive dialysis (36 h) into 50 mM Tris HCl (pH 7.2), 10% (w:v) glycerol, 0.1 mM EGTA and 1 mM DTT. After dialysis, free ATP represented less than 5% of the total ^{32}P label. Casein was prepared in essentially the same manner except that the catalytic subunit of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (rather than phosphorylase kinase) was used to phosphorylate the substrate.

PP activities were measured by incubation of the ^{32}P-labelled substrate (casein or phosphorylase) with extracts of purified HLMC at 37°C in the dialysis buffer in either the absence or the presence of PP inhibitors. PP inhibitors (okadaic acid, okadaol and okadaone) were added to HLMC extracts just before the addition of radiolabelled substrate. When inhibitor 2 was used, HLMC extracts were incubated for 15 min with inhibitor 2 before the addition of substrate (Cohen

et al., 1989). Aliquots of the incubation mixture were removed at 0, 7 and 14 min and added to ice-cold 25% TCA to which BSA (0.3 mg ml^{-1}) was added to aid protein precipitation. After centrifugation (13000 × *g*, 3 min), duplicate aliquots of the supernatant, containing the released ^{32}P, were added to liquid scintillant (Optiphase, Fisons, Loughborough, U.K.) and counted in a Beckman LS 5000 SE liquid scintillation counter. PP activity in extracts of HLMC was routinely assayed in a total reaction volume of 80 μl. HLMC extracts, containing 5×10^6 HLMC equivalents per 100 μl, were diluted either 1 in 80 for the assessment of phosphorylase PP activity or 1 in 20 for the assessment of casein PP activity.

To assess the effects of pretreatment of intact HLMC with okadaic acid on subsequent phosphorylase PP activity in extracts, the assay volume used was reduced (45 μl) in order to minimise dilution of the extract and PP activity was assessed in HLMC extracts diluted either 1 in 5 or 1 in 50. The dephosphorylation of phosphorylase displayed non-linear kinetics over 14 min when concentrated (1 in 5) extracts were used. However, linear kinetics were observed over shorter time periods. The liberation of ^{32}P from radiolabelled phosphorylase was, therefore, determined at 0, 1 and 2 min when concentrated (1 in 5) extracts were used and at 0, 7 and 14 min when more dilute (1 in 50) extracts were used.

Materials

The following were purchased from the sources indicated: dimethylsulphoxide (DMSO), sheep anti-human IgE, calcium ionophore A23187, Percoll, BSA, casein, HSA, EGTA, adenosine 5′-triphosphate (ATP), phosphorylase kinase, glycogen phosphorylase b, dithiothreitol (DTT), aprotinin, phenylmethylsulphonylfluoride (PMSF), leupeptin and soybean trypsin inhibitor (Sigma Chemical Co., Poole, U.K.); EDTA, calcium chloride and magnesium chloride (BDH, Poole, U.K.); gentamicin and penicillin-streptomycin (Gibco BRL, Dundee, U.K.); okadaic acid, okadaone and okadaol (LC laboratories, Nottingham, U.K.); Tris (Bio-Rad, Hemel Hempstead, U.K.); recombinant human inhibitor 2 (New England Biolabs, Hertfordshire, U.K.); monoclonal (IgG_1) mouse anti-human c-kit (Immunotech, Marseilles, France); magnetic beads coated with rat anti-mouse IgG_1 antibody (Dynal, Wirral, U.K.); EIA kits for the sulphopeptidoleukotrienes (sLT), leukotriene C_4 (LTC_4), LTD_4, LTE_4 (Amersham, Little Chalfont, U.K.); [$\gamma^{32}P$]-ATP (ICN Biomedicals, Thame, U.K.); EIA for prostaglandin D_2 (PGD_2) (Cayman Chemical Co. Michigan, U.S.A.).

Buffers

−PBS contained (mM): NaCl 137, $Na_2HPO_4\,12H_2O$ 8, KCl 2.7, KH_2PO_4 1.5. PBS was −PBS which additionally contained: $CaCl_2.2H_2O$ 1 mM; $MgCl_2.6H_2O$ 1 mM, glucose 5.6 mM, BSA 1 mg ml^{-1}, DNase 15 μg ml^{-1}. +PBS was −PBS supplemented with : $CaCl_2.2H_2O$ 1 mM, $MgCl_2.6H_2O$ 1 mM, glucose 5.6 mM, HSA 30 μg ml^{-1}. PBS-EDTA was −PBS supplemented with EDTA (1 mM). The pH of all PBS buffers was titrated to 7.3.

Hypotonic lysis buffer contained: Tris 50 mM, EDTA 1 mM, EGTA 0.1 mM, DTT 0.5 mM, PMSF 50 μg ml^{-1}, soybean trypsin inhibitor 50 μg ml^{-1}, leupeptin 5 μg ml^{-1} and aprotinin 5 μg ml^{-1}. The pH was titrated to 8.0.

Preparation of inhibitors and stimuli

Okadaic acid, okadaone and okadaol were prepared as 0.5 mM solutions in 10% DMSO. Ionophore A23187 was prepared as a 5 mM solution in absolute ethanol. Lyophilised polyclonal goat anti-human IgE antibody, was reconstituted in distilled water. All stock solutions were stored at −20°C with the exception of anti-IgE which was stored at 4°C. The drugs were diluted to the desired concentration in buffer just before use.

Statistics

Control and drug treated data sets were compared by Student's *t* test for paired data. Values of $P < 0.05$ were considered statistically significant.

Results

We have previously shown that okadaic acid inhibits the IgE-mediated release of mediators from HLMC (Peachell & Munday, 1993). In the present study, the effects of okadaic acid on the release of histamine and the generation of both sulphopeptidoleukotrienes (sLT) and prostaglandin D_2 (PGD_2) from HLMC was assessed. Okadaic acid inhibited the release of all three mediators in a dose-dependent manner and approximate IC_{50} values for the inhibition of the release of histamine, PGD_2 and sLT were 0.2, 0.2 and 0.6 μM, respectively (Figure 1). Two structural analogues of okadaic acid, okadaol and okadaone were also assessed for effects on the IgE-triggered release of mediators from HLMC (Table 1). Okadaic acid, okadaol and okadaone, in that order of decreasing activity, attenuated the release of histamine, PGD_2 and sLT from stimulated HLMC.

Our own previous studies (Peachell & Munday, 1993) have determined that the inhibition of mediator release from HLMC by okadaic acid is time-dependent with maximal inhibitory effects observed following a 2 h incubation with the inhibitor. In order to determine whether the requirement for lengthy incubation periods for maximal inhibition was due to difficulties okadaic acid may experience in entering HLMC, HLMC were incubated briefly (15 min) with okadaic acid (1 μM), the cells washed and then incubated for a further 105 min in the absence of okadaic acid before challenge with anti-IgE (Figure 2). Under these conditions, okadaic acid inhibited histamine release to a level approaching that seen when HLMC had been exposed to okadaic acid continuously for 120 min.

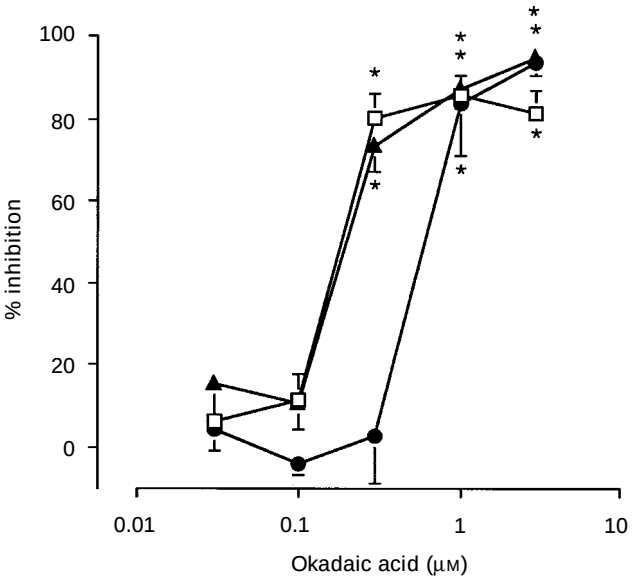

Figure 1 The effect of okadaic acid on the IgE-dependent generation of histamine, sulphopeptidoleukotrienes (sLT) and prostaglandin D_2 (PGD_2) from HLMC. Cells were incubated (2 h) with either buffer (control) or okadaic acid and then challenged with anti-IgE (1:300). Mediator release was allowed to proceed for a further 25 min. Results are expressed as % inhibition of the control mediator releases which were: histamine (□), 26 ± 7%; sLT (●), 5 ± 1.7 ng per 10^6 HLMC; PGD_2 (▲) 258 ± 88 ng per 10^6 HLMC. Values are the means and vertical lines s.e.mean, $n = 5$. Statistically significant inhibition ($P < 0.05$) of mediator release is denoted by an asterisk.

Table 1 Inhibition of mediator release from HLMC by okadaic acid and analogues

	% inhibition		
	Histamine	*sLT*	*PGD_2*
Okadaic acid	82±5*	94±3*	95±3*
Okadaol	47±8*	51±9*	44±7*
Okadaone	6±3	15±8	16±7

The effect of okadaic acid and analogues of okadaic acid on the IgE-dependent generation of histamine, sLT and PGD_2 from HLMC. Cells were incubated (2 h) in either buffer (control) okadaic acid, okadaol or okadaone (all at 3 μM) and then challenged with anti-IgE (1:300). Mediator release was allowed to proceed for a further 25 min. Results are expressed as % inhibition of the control mediator releases which were: histamine, 38±7%; sLT, 5±1.7 ng per 10^6 HLMC, PGD_2, 264±50 ng per 10^6 HLMC. Values are the means±s.e.mean, $n=5$. Statistically significant ($P<0.05$) inhibition is denoted by an asterisk.

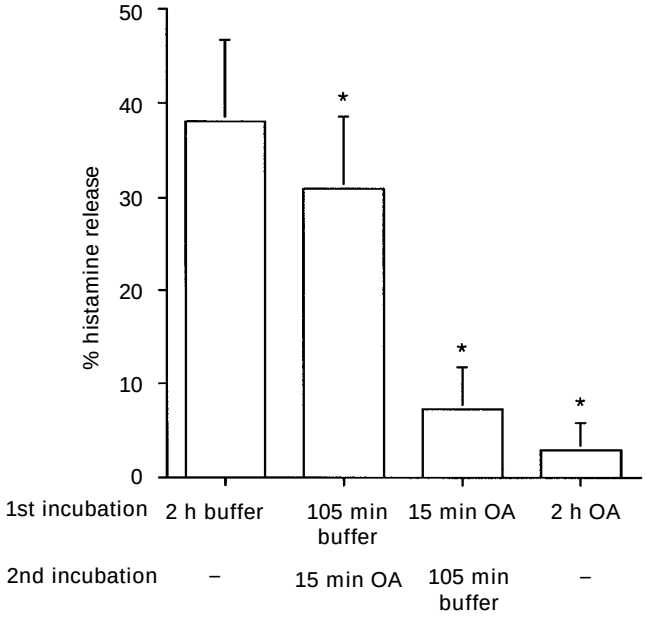

Figure 2 Time-dependence of the okadaic acid inhibition of anti-IgE-induced histamine release from HLMC. HLMC were either, (i) pretreated for 15 min with okadaic acid (OA, 1 μM), washed and incubated for 105 min in buffer, (ii) incubated for 105 min in buffer and then for 15 min with okadaic acid (1 μM), (iii) incubated for 120 min with okadaic acid (1 μM) or, (iv) incubated in buffer for 120 min. HLMC subjected to all pretreatments were then challenged with anti-IgE (1:300). Histamine release was allowed to proceed for a further 25 min. Values are the means±s.e.mean, $n=4$. Statistically significant ($P<0.05$) reduction of control histamine release is denoted by an asterisk.

The inhibitory effects of okadaic acid on HLMC were irreversible over 20 h (Figure 3). If HLMC were incubated with okadaic acid for 1 h, washed and then maintained in buffer for 20 h, then the level of inhibition (91±2%) of anti-IgE-induced histamine release was similar to that seen when okadaic acid had been present continuously for 21 h (inhibition after 21 h incubation with okadaic acid, 99±1%; inhibition after 1 h incubation with okadaic acid, 68±13%, $n=6$). Qualitatively similar data were observed when the same experiment was performed but ionophore A23187 was used as the stimulus rather than anti-IgE (data not shown). The data indicate that the continuous extracellular presence of okadaic acid is not necessary to obtain inhibitory effects in HLMC and that the inhibitory effects of okadaic acid persist for 20 h after removal of the cells from okadaic acid.

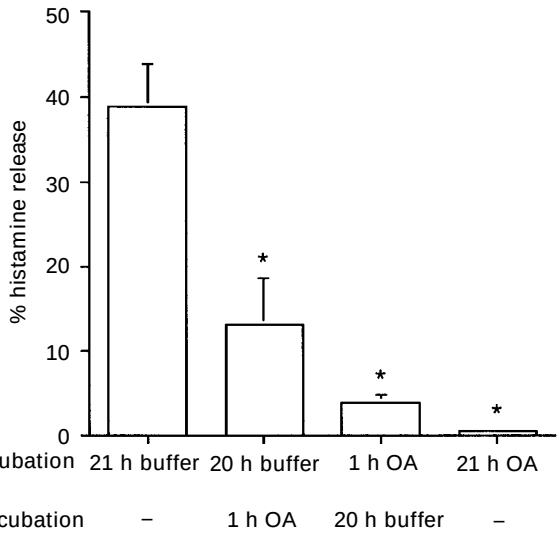

Figure 3 The persistence of okadaic acid inhibition of anti-IgE-induced histamine release from HLMC. HLMC were either, (i) pretreated for 1 h with okadaic acid (OA, 1 μM), washed and incubated for 20 h in buffer, (ii) incubated for 20 h in buffer then for 1 h with okadaic acid (1 μM), (iii) incubated for 21 h with okadaic acid (1 μM) or, (iv) incubated in buffer for 21 h. HLMC subjected to all treatments were then challenged with anti-IgE (1:300). Histamine release was allowed to proceed for a further 25 min. Values are the means±s.e.mean, $n=6$. Statistically significant ($P<0.01$) reduction of control histamine release is denoted by an asterisk.

Our previous studies (Peachell & Munday, 1993) indicated that extracts of purified HLMC liberate ^{32}P from radiolabelled glycogen phosphorylase and that okadaic acid inhibits this PP activity. In the present study, extracts of purified HLMC were prepared and incubated with either, okadaic acid, okadaol or okadaone and the effects on the dephosphorylation of glycogen phosphorylase determined (Figure 4). The data indicate that okadaic acid, okadaol and okadaone, in that order of decreasing activity, inhibited PP activity present in extracts of HLMC. In these same experiments, the effects of pretreatment (2 h) with okadaic acid, okadaol and okadaone on anti-IgE-induced histamine release from HLMC were determined in parallel. There was a good correlation between the extent of inhibition of histamine release and the attenuation of PP activity for a given PP inhibitor.

The effects of pretreatment of HLMC with a PP inhibitor on subsequent PP activity in extracts of HLMC was assessed. In preliminary experiments, PP activity in extracts prepared from HLMC which had been pretreated (2 h) with okadaic acid (3 μM) was not significantly different from that in extracts of untreated cells. However, this lack of effect may have been due to the manner in which these experiments were performed. Usually, HLMC extracts were diluted (1 in 80) in order to obtain linear rates of dephosphorylation over 14 min. We hypothesize that the act of diluting the extracts may also serve to dilute any okadaic acid that may have been trapped in the cells during the pretreatment step. To test this, HLMC were preincubated for 2 h with or without okadaic acid (3 μM) after which time extracts of the pretreated HLMC were prepared for use in PP assays. Dilutions (1 in 5 or 1 in 50) of the same HLMC extracts were assayed for PP activity. The dephosphorylation of radiolabelled phosphorylase by the more dilute (1 in 50) extracts was assessed over 14 min. Assays of PP activity in the more concentrated (1 in 5) extracts contained a greater quantity of cellular material and thus a higher level of PP activity. In order to retain linear rates of dephosphorylation the time course of the liberation of ^{32}P from radiolabelled phosphorylase by more concentrated HLMC extracts was followed over 2 min. Whereas an effect of okadaic acid pretreatment was evident in the more concentrated pre-

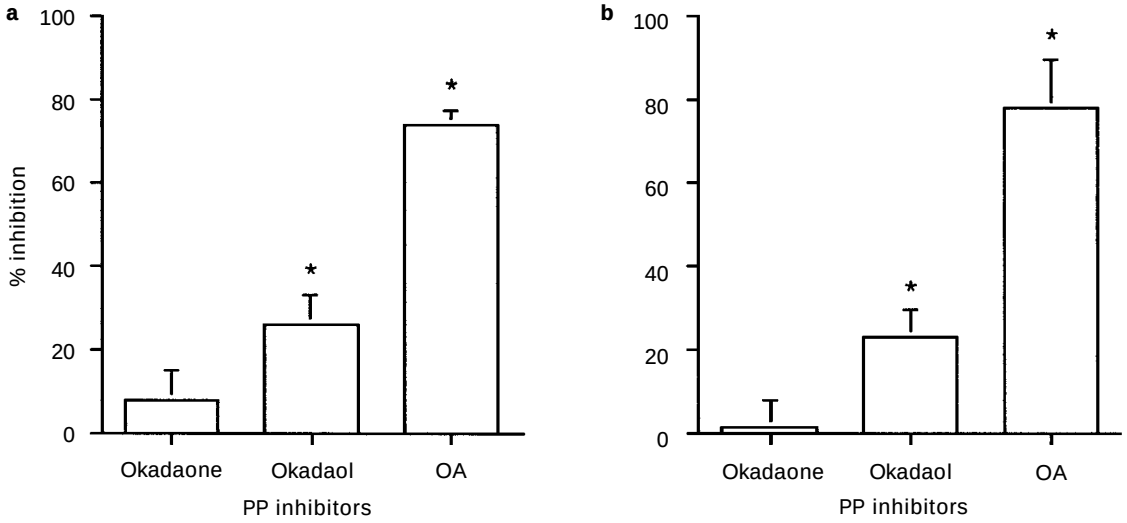

Figure 4 The effect of okadaic acid (OA) and analogues of okadaic acid, okadaone and okadaol, on (a) phosphatase (PP) activity and (b) histamine release from HLMC. (a) The liberation of ^{32}P from radiolabelled glycogen phosphorylase was monitored at 0,7 and 14 min in the presence or absence of a PP inhibitor (3 μM). Kinetic data were reworked to give rates of dephosphorylation in the presence or absence of PP inhibitors. The results are expressed as % inhibition of the control PP activity (76 ± 17 pmol ^{32}P per 10^6 HLMC per min). (b) The effect of PP inhibitors on histamine release was assessed in parallel. HLMC were incubated (2 h) with a PP inhibitor (3 μM) and then challenged with anti-IgE (1:300) for a further 25 min for histamine release. Results are expressed as % inhibition of control histamine release which was 30 ± 4%. Statistically significant ($P < 0.05$) inhibition of either PP activity or histamine release is denoted by an asterisk. All values are the means $\pm$ s.e.mean, $n = 4$. HLMC preparations of 75 ± 4% purity were generated by CCE.

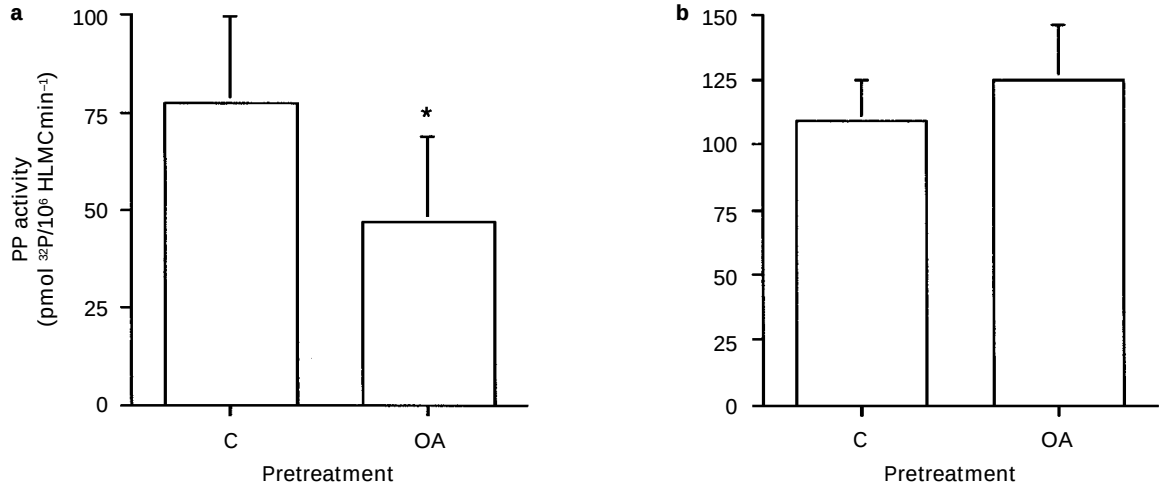

Figure 5 Dephosphorylation of radiolabelled glycogen phosphorylase by (a), concentrated and (b), diluted extracts of purified HLMC pretreated with okadaic acid. Purified HLMC were incubated (2 h) in either buffer (control, C) or okadaic acid (OA, 3 μM) and extracts prepared. HLMC extracts were used either in a concentrated (a) or in a diluted (b) form. The liberation of ^{32}P from radiolabelled phosphorylase by concentrated extracts (diluted 1:5 by the addition of substrate) was monitored at 0, 1 and 2 min. The liberation of ^{32}P from radiolabelled phosphorylase by diluted extracts (diluted 1 in 50) was monitored at 0, 7 and 14 min. The kinetic data were reworked to determine overall rates of dephosphorylation and these values are presented in the figure. Statistically significant ($P < 0.05$) inhibition of control PP activity is denoted by an asterisk. Values represent the means $\pm$ s.e.mean, $n = 3$. HLMC preparations of 74, 84 and 93% purity were generated by CCE.

paration (46 ± 12% inhibition; Figure 5a) no effect on dephosphorylation was observed in the more dilute preparation (Figure 5b). In these experiments, histamine release was assessed in parallel, and pretreatment (2 h) with okadaic acid (3 μM) inhibited histamine release by 90 ± 6%.

Previous studies (Cohen *et al*., 1989) have shown that a low (2 nM) concentration of okadaic acid inhibits PP2A selectively whereas a higher (5 μM) concentration inhibits both PP1 and PP2A. In a total of five experiments in which glycogen phosphorylase was used as the substrate, 2 nM okadaic acid inhibited PP activity in HLMC extracts by 15 ± 3% ($P < 0.001$) and 5 μM okadaic acid inhibited PP activity by 88 ± 4% ($P < 0.0005$). These data suggest that 73% of the total PP activity is due to PP1 and 15% due to PP2A. In three of these five experiments (Figure 6), the effects of recombinant inhibitor 2, a selective inhibitor of PP1, were also studied. At a concentration (20 nM) that has been shown to be maximally effective against PP1 (Honkanen *et al*., 1991), inhibitor 2 was found to attenuate PP activity in extracts of HLMC by 71 ± 3% ($P < 0.005$, $n = 3$). In combination with 2 nM okadaic acid, inhibitor 2 attenuated PP activity by 86 ± 4% ($P < 0.005$, $n = 3$). These data suggest that 71% of the total PP activity is due to PP1 and 15% is due to PP2A. Extracts of purified HLMC (91 ± 3% purity, $n = 3$) also dephosphorylated radiolabelled casein, a PP2A-restricted substrate if magnesium ions are not present (Agostinis *et al*., 1987), and this PP activity was inhibited (81 ± 8% inhibition, $P < 0.0005$, $n = 6$) by 2 nM okadaic acid yet unaffected by inhibitor 2 (20 nM).

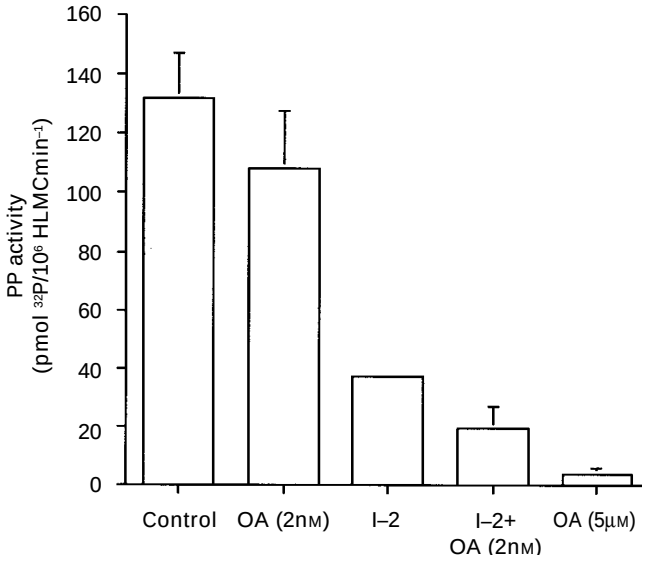

Figure 6 Effect of okadaic acid and inhibitor 2 on phosphatase (PP) activity in extracts of HLMC. Dephosphorylation of radiolabelled glycogen phosphorylase was followed over 14 min and results are expressed as the rate of dephosphorylation in either the absence or the presence of a PP inhibitor. Extracts of HLMC were prepared and then incubated with either a low (2 nM) or a higher (5 μM) concentration of okadaic acid (OA) and effects on dephosphorylation determined. Alternatively, extracts were incubated with inhibitor 2 (I-2) (20 nM) either with or without okadaic acid (2 nM) and effects on dephosphorylation established. Results are expressed as means $\pm$ s.e.-mean, $n=3$ with HLMC preparations of $93\pm2\%$ purity. HLMC were purified by immunomagnetic bead separations.

Discussion

The importance of PPs and of protein dephosphorylation in a variety of cellular processes has been highlighted by the use of cell-permeant PP inhibitors such as okadaic acid in intact cells (Cohen *et al*., 1990; Hardie *et al*., 1991; Hardie, 1993). In the present study, we investigated a potential role for PPs in the regulation of HLMC function by use of okadaic acid and additional PP inhibitors.

Okadaic acid inhibited the IgE-dependent release of histamine, and the generation of both PGD_2 and sLT in a dose-dependent manner. Okadaic acid was equipotent (IC_{50}, 0.2 μM) as an inhibitor of histamine release and PGD_2 generation suggesting that okadaic acid acts at a common target to modulate the production of either mediator. However, okadaic acid was less potent (IC_{50}, 0.6 μM) as an inhibitor of the generation of sLT which may suggest that the pathway leading to sLT generation is less susceptible to modulation by okadaic acid than those leading to either histamine release or PGD_2 synthesis.

We have previously shown that a 2 h incubation with okadaic acid is required in order to obtain optimal inhibition of histamine release from activated HLMC (Peachell & Munday, 1993). In the present study, it was established that the continuous extracellular presence of okadaic acid over 2 h was not required in order to obtain optimal inhibitory effects. Thus a brief (15 min) incubation of HLMC with okadaic acid followed by incubation (105 min) in the absence of okadaic acid was as effective at inhibiting histamine release as a continuous incubation of HLMC with okadaic acid over 2 h. These data suggest that okadaic acid acts intracellularly and, moreover, that okadaic acid enters the cell quite readily but that the process (or processes) that okadaic acid modulates takes some time. Should the intracellular target of okadaic acid be a PP, then it is possible that okadaic acid may act to enhance the phosphorylation of a target protein by a protein kinase. The interaction of okadaic acid with a PP may occur quite rapidly but if the kinase has a slow turnover rate, then the effects on mediator release may take some time to be seen. Interestingly, the effects of okadaic acid on HLMC were not reversed even 20 h after the cells had been removed from okadaic acid. Whether this reflects a sustained and dynamic interaction of okadaic acid at a target or an eventual irreversible inactivation of the HLMC is not known.

Okadaic acid, okadaol and okadaone have previously been shown to inhibit the stimulated release of histamine from HLMC (Peachell & Munday, 1993). In the present study, the effects of these compounds on the generation of sLT and PGD_2 were found to parallel the effects observed on histamine release. The rank order of inhibition of the release of mediators of okadaic acid > okadaol > okadaone parallels the action of these compounds as inhibitors of PP activity (Nishiwaki *et al*., 1990). These data suggest that okadaic acid and analogues of okadaic acid act to inhibit mediator release by interacting with PPs. Indeed, further studies to assess the effects of these compounds on PP activities in extracts of HLMC indicate that a very good correlation exists between the extent of inhibition of histamine release and the attenuation of PP activity. However, it should be stressed that, in these experiments (Figure 4), HLMC were pretreated (2 h) with a PP inhibitor for the mediator release component of the experiment whereas to assess PP activities, extracts were prepared and then PP inhibitors added to the extracts. Therefore, a direct comparison of the effects of PP inhibitors on mediator release and PP activity may not be strictly valid.

Studies were performed to establish the effect of pretreatment (2 h) of HLMC with okadaic acid on subsequent PP activity in extracts. In initial experiments, pretreatment of HLMC had no effect on the PP activity in subsequently-prepared extracts compared to untreated HLMC. In these experiments, HLMC extracts were diluted (1 in 80) in order to obtain linear rates of dephosphorylation over 14 min. However, the dilution of extracts may have also diluted any okadaic acid that may have been present in the cells after pretreatment. If, however, more concentrated extracts (1 in 5) were prepared and kinetics followed over a shorter time period (2 min), in order to preserve linear rates of dephosphorylation, then pretreatment with okadaic acid did attenuate ($46\pm12\%$ inhibition) PP activity in HLMC extracts but this level of inhibition did not correspond with the extent of inhibition ($90\pm6\%$ inhibition) of histamine release. Parenthetically, it should also be noted that the preparation of a cell extract will serve to dilute the intracellular contents of the cell. Thus, even before experimental dilutions (i.e. 1 in 5, 1 in 50 or 1 in 80) are considered, the neat extract itself is a dilution. These considerations indicate that the extent of inhibition of PP activity in extracts of cells previously pretreated with okadaic acid cannot provide information on the extent of inhibition of PP activity in the intact cell.

Studies of PP activities in extracts of HLMC suggest that both PP1 and PP2A are present in HLMC. Several approaches were adopted to determine whether PP1 and PP2A are present in HLMC. The methods involved the use of a low (2 nM) concentration of okadaic acid, which should inhibit PP2A selectively (Cohen *et al*., 1989), and a higher (5 μM) concentration of okadaic acid, which should inhibit both PP1 and PP2A (Cohen *et al*., 1989). Alternatively, the use of inhibitor 2, a PP1-selective inhibitor, in either the presence or the absence of a low (2 nM) concentration of okadaic acid was evaluated. Either approach generated very similar results indicating that PP1 contributes approximately 72% of the total PP activity in extracts of HLMC and PP2A about 15% of the activity. Although these data imply that HLMC contain approximately five times more PP1 than PP2A, these experiments probably underestimate the relative contribution of PP2A to the total PP activity because PP2A is several fold less active against the substrate (glycogen phosphorylase) used in these experiments (Tung *et al*., 1985). Confirmation that HLMC contain PP2A was provided by the finding that extracts of HLMC dephosphorylate radiolabelled casein, a PP2A-restricted substrate if

magnesium ions are not present (Agostinis *et al*., 1987). That this casein PP activity was inhibited by 2 nM okadaic acid further suggests the involvement of PP2A. Taken together, these data indicate that both PP1 and PP2A are present in HLMC but the relative contributions of these PPs to the total PP activity cannot be determined with certainty.

Although the data suggest that both PP1 and PP2A are probably present in HLMC it is not possible to determine whether either PP1 or PP2A or whether both PPs are involved in the regulation of HLMC responses. The IC_{50} (200 nM) for the inhibition of histamine release is between three and twenty fold higher than the IC_{50} (10–70 nM) found for the inhibition of PP1 (Bialojan & Takai, 1988; Cohen *et al*., 1989). That high concentrations of okadaic acid are required to inhibit histamine release could suggest that PP1 is the more important PP activity with respect to the inhibition of mediator release from HLMC by okadaic acid. However, because the interaction between okadaic acid and PP2A approaches stoichiometry (Cohen *et al*., 1989), and because the concentration of PP2A in cells could approach micromolar concentrations (Hardie *et al*., 1991; Hardie, 1993), micromolar concentrations of okadaic acid may be necessary to inhibit intracellular PP2A. These considerations highlight the difficulties associated with attributing the inhibition of mediator release by okadaic acid to an interaction with a given PP.

The present work has perhaps given the misleading impression that PPs other than PP1 and PP2A are not involved in regulating the response of HLMC. Certainly, evidence has been obtained that PP2B (calcineurin) may regulate HLMC responses based on data with cyclosporin and FK 506 both of which are known to inhibit PP2B (Liu *et al*., 1991) and both of which inhibit mediator release from HLMC (Triggiani *et al*., 1989; de Paulis *et al*., 1991). Moreover, the possibility that alternative PPs could contribute to the regulation of HLMC responses cannot be excluded.

The present work has provided evidence that both PP1 and PP2A are resident in HLMC. Furthermore, the data indicate that okadaic acid inhibits mediator release from HLMC and that the mechanism of inhibition more than likely involves the attenuation of PP activities. This raises the possibility that PPs could serve as targets for the development of agents with therapeutic potential to prevent the potentially deleterious consequences of mediator release from HLMC.

The authors are grateful to Mr N. Saunders and Mr R. Nair (Cardiothoracic Surgery) and Dr P. DaCosta (Histopathology) at the Seacroft/Killingbeck Hospitals, Leeds; to Mr A. Thorpe (Cardiothoracic Surgery) and Dr K. Suvarna and Dr A. Kennedy (Histopathology) at the Northern General Hospital, Sheffield for their invaluable help in providing lung tissue specimens. This work is supported by the National Asthma Campaign and the Wellcome Trust.

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(Received July 19, 1996
Revised October 17, 1996
Accepted October 22, 1996)